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Production of waxy (amylose-free) wheats

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Abstract The Waxy (Wx) protein has been identified as granule-bound starch synthase (GBSS; EC 24.1.21), which is involved in amylose synthesis in plants. Although common wheat (*Triticum aestivum* L.) has three Wx proteins, "partial waxy mutants" lacking one or two of the three proteins have been found. Using such partial waxy mutants, tetra- and hexaploid waxy mutants with endosperms that are stained red-brown by iodine were produced. Both mutants showed loss of Wx protein and amylose. This is the first demonstration of genetic modification of wheat starch.

Key words Wheat · Waxy · Amylose · Wx protein · Mutant

Introduction

Recently, many genes encoding enzymes involved in starch synthesis have been cloned and used for genetic manipulation (Visser and Jacobsen 1993; Smith and Martin 1993; Müller-Röber and Koßmann 1994). One of these enzymes, granule-bound starch synthase (GBSS; EC 24.1.21), is the key enzyme in amylose synthesis (Preiss 1991). GBSS binds tightly to starch granules and is known as Wx protein (Echt and Schwartz 1981; Vos-Scheperkeuter et al. 1986; Preiss 1991). Amylose and amylopectin are the two major polysaccharide components of reserve starch in plants. The amylose content in total reserve starch varies from 11%–37% in nonmutant phenotypes (Shannon and Garwood 1984), whereas "waxy" (amylose free or glutinous) mutants have essentially no amylose. Waxy mutants also lack Wx protein, with the exception of several maize waxy mutants (Echt and Schwarz 1981). Nonwaxy and waxy phenotypes are easily distinguishable by staining of starch with iodine. Nonwaxy starch stains blue-black while waxy starch stains red-brown.

Waxy mutants have been identified in cereals such as maize, rice, barley, sorghum and amaranth. However, waxy mutants have not been reported in tetra- or hexaploid wheat. Common wheat has three homoeologous *waxy* genes, *WX-A1*, *WX-B1* and *WX-D1*, located on the group 7 chromosomes (Chao et al. 1989). It was thought likely that these genes produce at least three GBSS isozymes, as found for many wheat homoeologous gene-encoded functional proteins (Hart 1987). Indeed a modified two-dimensional gel electrophoresis (2D-PAGE) method (Nakamura et al. 1993a) enabled us to identify three Wx proteins, Wx-A1, Wx-B1 and Wx-D, which have slightly different molecular weights and/or isoelectric points (Fig. 1). Using this technique, we were able to identify mutants (Nakamura et al. 1993b, c; Yamamori et al. 1994), each lacking one or two Wx proteins, which were termed "partial waxy mutants". Wild type was designated Type 1, Type 2 is lacking in Wx-A1 protein, Type 3 in Wx-B1, Type 4 in Wx-D1 and Type 7 in both Wx-A1 and Wx-B1 (Fig. 2). In this study, we describe the production of waxy tetra- and hexaploid wheats through the use of these mutants. Since wheat is one of the most widely cultivated crops in the world and its starch is used in food and nonfood industries, waxy wheats will have important implications in both fields.

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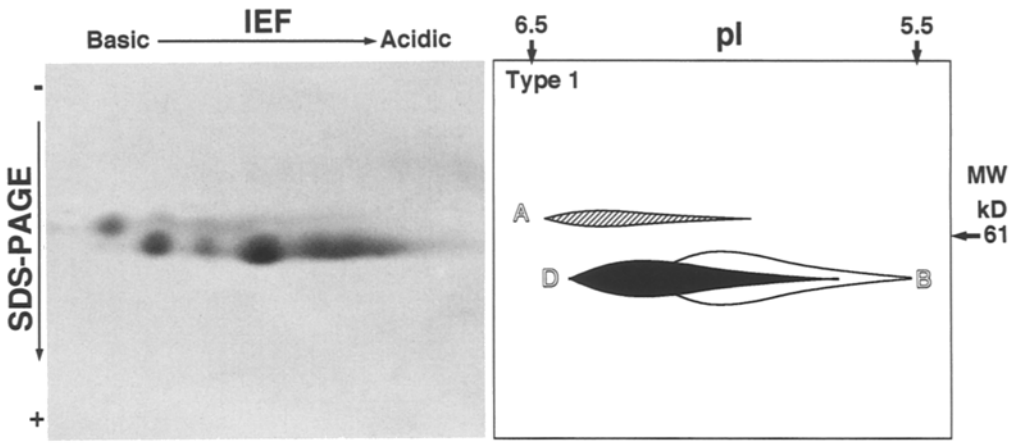

Fig. 1 Two-dimensional gel electrophoresis pattern of Wx protein of common wheat (*Triticum aestivum* L.) (left panel) and a diagrammatic representation of the pattern (right panel). The diagrams show differences in molecular weight (*MW*), isoelectric point (*pI*), and amount (size of the torpedo shapes) of three Wx proteins. *A*, *B* and *D* in the diagram indicate the three Wx proteins Wx-A1, Wx-B1 and Wx-D1, respectively. The normal phenotype has the three Wx proteins and is designated Type 1

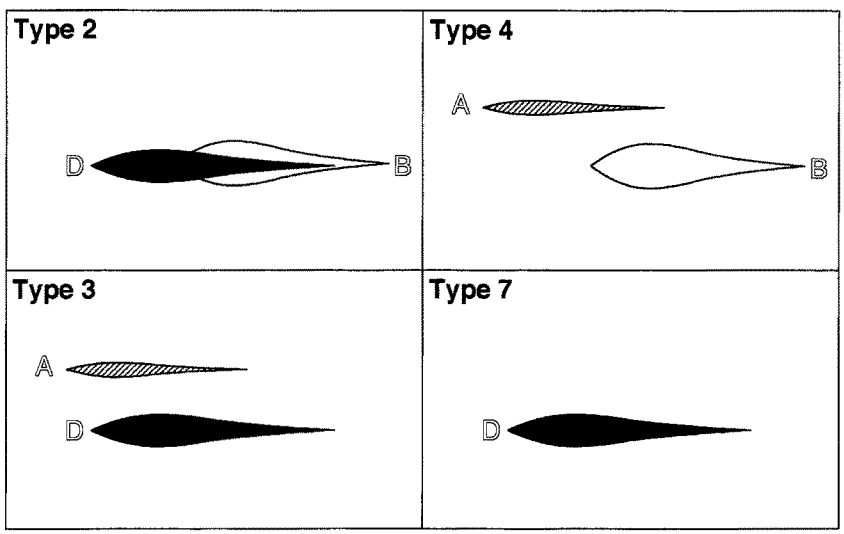

Fig. 2 Schematic diagrams of Wx protein patterns in partial waxy mutants. The Type 2 mutant lacks Wx-A1 protein, Type 3 lacks Wx-B1 protein, Type 4 lacks Wx-D1 protein, and Type 7 lacks both Wx-A1 and Wx-B1 proteins

Materials and methods

Plant materials

Two common wheats, Kanto 107 (K107) and Bai Huo, and a durum wheat (*Triticum durum* Desf.) cv. Aldura were used. K107 is a Type 7 mutant and Bai Huo is a Type 4 mutant (Yamamori et al. 1994; Fig. 2). Aldura has both Wx-A1 and Wx-B1 proteins. Embryos of the F_2 seeds of K107 × Aldura, in which endosperms stained red-brown with iodine, were sterilized, excised and germinated on MS medium without hormone. At the three-leaf stage, the plants were transplanted into horticultural soil (Kureha) and grown in a greenhouse at 21° C(day)/15° C(night).

Starch granule preparation

Starch granules were prepared as described previously (Nakamura et al. 1993a). The starch granules were purified using SDS buffer containing 60 mM TRIS-HCl, pH 6.8, 3% (w/v) SDS, 3% (v/v) β-mercaptoethanol and 10% (v/v) glycerol. All the steps in the preparation were performed at 4° C. Purified starch granules were stored at − 20° C.

Extraction of Wx protein and electrophoresis

Protein extraction for SDS-PAGE was performed as described by Echt and Schwartz (1981). For 2D-PAGE, 10 mg of the prepared starch granules were mixed with 300 µl of lysis buffer [8 M urea, 2% (v/v) Nonidet-P40, 2% (v/v) Ampholine pH 3.5 ~ 10, 5% (v/v) β-mercaptoethanol and 5% (w/v) polyvinylpyrrolidone] and heated in a boiling water bath for 2 min, then cooled on ice. SDS-PAGE was performed by the system of Laemmli (1970) using 10% acrylamide gels and 2D-PAGE was performed as previously reported (Nakamura et al. 1933a). After electrophoresis, gels were stained with a silver staining kit (Wako Pure Chemicals) or Coomassie Brilliant Blue R.

Iodine staining

One month after anthesis, fresh seeds were harvested, endosperms were cut into halves, and one-half was stained by iodine solution (740 mg resublimed iodine and 1480 mg potassium iodine dissolved in 400 ml deionized distilled water). Starch granules were also stained with this solution and analyzed microscopically.

Table 1 Characteristics of waxy tetraploid wheats and their parents

Plant	Chromosome number	KI/I_2 staining of endosperms			Amylose content (%)	Wx protein
		No. of samples	Blue-black	Red-brown		
K107	42	100	100	0	25.9	+
Aldura	28	100	100	0	36.3	+
K107 × Aldura						
F_1	35	288	284	4[a]		(−)[a]
F_2 S-1	29	221	0	221	0.7	−
S-2	29	178	0	221	0.7	−
5-2	30	507	0	507	0.7	−
25-2	29	88	0	88	0.7	−
F_3[b] S-1-2	28	882	0	882	0.6	−
S-1-3	28	1215	0	1215	0.6	−
S-1-5	28	1013	0	1013	0.6	−
Waxy maize					0.6	−

[a] Wx protein of the four seeds was analyzed
[b] S-1-2, S-1-3 and S-1-5 were randomly chosen from F_3 progeny from F_2 plant, S-1

Chromosome number

Immature embryos were germinated under sterile conditions on MS medium without hormone. Root tips were excised, treated for 24 h at 1° C in distilled water, fixed in Carnoy's fluid (ethanol : glacial acetic acid 3:1) for 48 h, and then stored at 4° C until examination. The root tips were hydrolyzed in 1 N HCl at 60° C for 10 to 15 min, stained with Feulgen regent for 1 h, and squashed with a coverslip in 45% acetic acid and observed.

Amylose content

Amylose content was measured colorimetrically using an autoanalyzer (Bran Lubbe). Fifty milligrams of starch granules extracted by the above method were suspended in 5 ml of a solution containing 0.75 N NaOH and 25% ethanol, incubated at 25° C for 2 h, and then applied to the autoanalyzer. Regression curves were derived using potato amylose and amylopectin (Sigma) as the standards (Yamamori et al. 1992).

Results

Type 7 × tetraploid (emmer) wheat

Crossing hexaploid wheat with tetraploid wheat is a common method of eliminating the D genome from hexaploid wheat and producing extracted tetraploid wheats (Kerber 1964). A hexaploid, Type 7 mutant, K107, was crossed with Aldura, a tetraploid. Elimination of the D genome from K107 allowed the production of waxy tetraploid wheat.

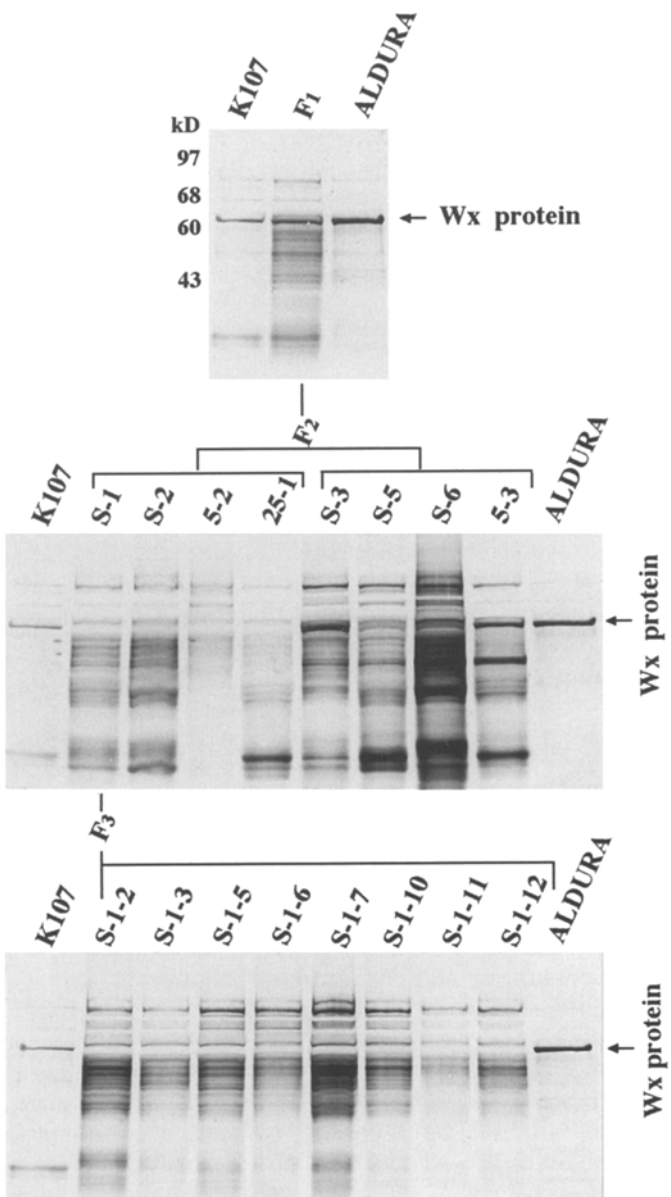

Fig. 3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of Wx protein in K107, Aldura and the progeny of this cross. S-1, S-2, 5-2 and 25-1 were F_2 seeds in which endosperm stained red-brown by iodine solution while S-3, S-5, S-6 and 5-3 were F_2 seeds with blue-black staining endosperm. All F_3 seeds were randomly chosen from F_2 parent S-1. In F_1, F_2 and F_3 seeds, Wx proteins were extracted from starch granules that were prepared from a single seed

Table 2 Segregation of waxy type seeds in F_2 seeds of K107 × Bai Huo

F_1 plant	Number of seeds	KI/I_2 staining of endosperms		χ^2 (63:1)	P
		Blue-black	Red-brown		
No. 1	556	549	7	0.33	0.50–0.80
No. 2	728	719	9	0.52	0.30–0.50
No. 3	511	504	7	0.12	0.50–0.80
Total	1795	1772	23		

Fig. 4 Endosperms stained with iodine solution. The F_2 seeds of K107 (Type 7) × Bai Huo (Type 4) included segregants with red-brown staining endosperm (waxy type)

Four seeds in which endosperms and starch granules stained red-brown by iodine segregated from 288 F_2 seeds matured on the F_1 plants (Table 1). There was no Wx protein in the starch granules of these four seeds. The nonwaxy F_2 seeds from this cross all had Wx protein, although the protein level varied (Fig. 3).

Chromosome numbers in the four F_2 plants were decreased from the 35 of the F_1 pentaploid to 29, 29, 30 and 29, respectively (Table 1). All F_3 seeds matured on the four F_2 plants showed the waxy phenotype and three randomly chosen F_3 plants all had a chromosome number of 28 (Table 1). N- and C-banding of chromosomes indicated that the D genome was absent in these plants (data not shown). The waxy phenotype and chromosome number of F_3 individuals was stably transmitted to the F_4 generation (Table 1).

The level of amylose (0.6%–0.7% of starch) in F_3 and F_4 seeds was comparable to that in a waxy maize (0.6%), although both parents had amylose contents greater than 25% (Table 1). Seed starch was analyzed by gel filtration using a Sephacryl S-1000 column. Only an amylopectin peak was seen in the mutant, whereas both parents showed amylopectin and amylose peaks (data not shown).

Type 7 × Type 4

Waxy hexaploid wheat could be produced by crossing Type 7 with Type 4 mutants. Based on Mendelian segregation, the F_2 generation should include individuals lacking all three Wx proteins. These plants should be waxy mutants, because mutants lacking Wx protein in various diploid plants are all waxy mutants (Echt and Schwartz 1981; Sano 1984; Konishi et al. 1985; Hovenkamp-Hermelink et al. 1987).

All F_2 seeds matured on the three F_1 plants of K107 × Bai Huo were harvested and endosperms were stained with iodine solution. Twenty-three seeds staining red-brown were identified among 1795 F_2 seeds

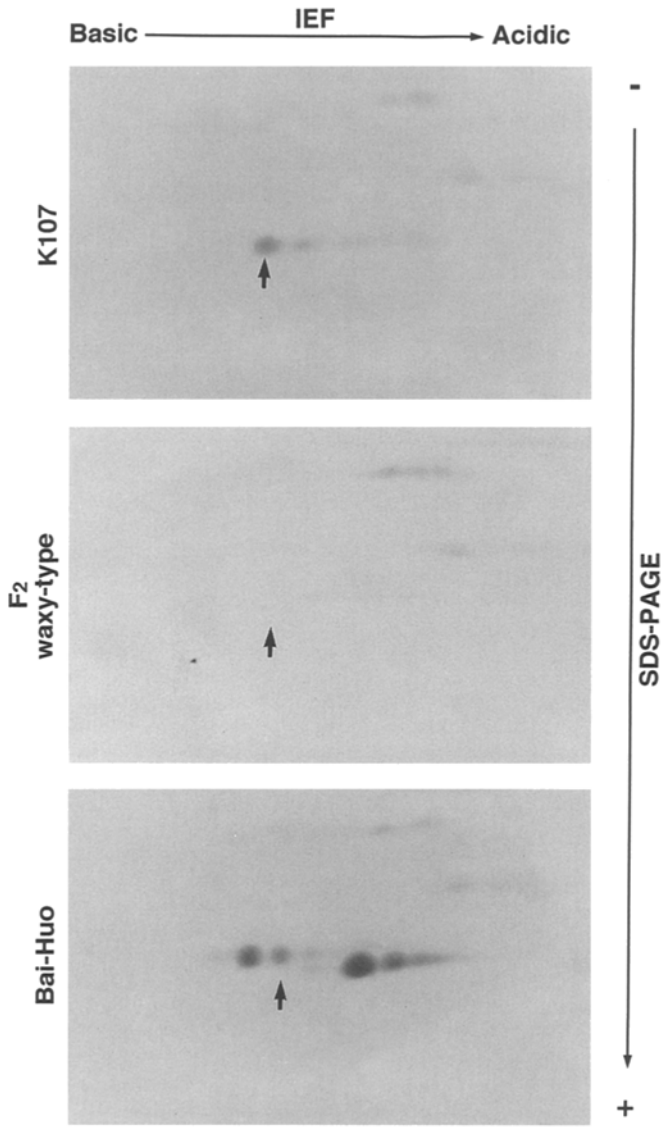

Fig. 5 Two-dimensional (2D)-PAGE analysis of Wx protein. K107 shows only Wx-D1 protein while Bai Huo shows Wx-A1 and Wx-B1 proteins. The F_2 individual that has red-brown staining endosperm (waxy type) lacks all three Wx proteins. *Arrows* indicate identical positions

(Table 2, Fig. 4). The Chi-square test result indicated that the observed frequencies were not significantly different from the expected 63:1 segregation ratio (Table 2). Therefore, while the synthesis of amylose in diploid plants, such as maize and rice, is controlled by one *waxy* gene, amylose synthesis in common wheat is controlled by three *waxy* genes.

The starch granules of the waxy type endosperms also lacked Wx protein, though K107 had Wx-D1 protein and Bai Huo had both Wx-A1 and Wx-B1 proteins (Fig. 5). Starch granules extracted from 23 F_2 seeds showed the same level of amylose (0.7%) as waxy tetraploid wheat. The physical and chemical properties of the new starch, as well as the genetic stability of the new starch phenotype, are being investigated in subsequent generations. Results obtained so far strongly support that the new phenotype produced in this study is waxy hexaploid wheat (manuscript in preparation).

Discussion

Two methods have been used to produce waxy mutants. One involves mutagenic treatments such as X-rays or ethylmethane-sulfonate (EMS). Using this method, waxy mutants have been produced in maize (Briggs et al. 1965) and rice (Amano 1981), and an *amf* mutant obtained in diploid potato (Hovenkamp-Hermelink et al. 1987). A waxy mutation was also reported in the diploid wheat *Triticum monococcum* (Kanzaki and Noda 1988). However, this method has proven unsuitable for producing waxy mutants in tetra- and hexaploid wheats because polyploid wheats have been shown to have a high resistance to mutagens, as revealed by a low frequency of mutants (Mackey 1964; Sears 1972; Yamashita, personal communication). The second method is gene manipulation using antisense technology, which was used to obtain transgenic plants with altered amylose/amylopectin ratios in potato (Visser et al. 1991) and rice (Shimada et al. 1993). It was anticipated that the antisense method would allow production of a transgenic wheat plant without amylose (Lazzeri and Shewry 1993) since two groups have obtained fertile transgenic plants from common wheats (Vasil et al. 1992; Weeks et al. 1993). However, identification of partial waxy mutants prompted us to employ the third simple but proven method of producing waxy wheats used in this study, which does not require plant transformation.

The D genome may suppress the expression of genes located on the A and B genomes in common wheat (Kerber and Green 1980; Galili and Feldman 1984). However, the inactivation of the *Wx-A1* and *Wx-B1* loci of K107 is not caused by D-genome suppression as the D genome was absent in waxy tetraploid wheat. To determine whether structural genes are present in the inactive *waxy* loci of partial waxy mutants, the functionally null alleles have been subjected to preliminary molecular analysis. Southern and polymerase chain reaction (PCR) results showed the presence of structural genes in the three *waxy* loci of K107 and Bai-huo (Nakamura et al. 1994). PCR analysis using internal primers from a *waxy* cDNA clone of wheat (Ainsworth et al. 1993) has shown that there is a small deletion (< 30 bp) in the *Wx-A1b* allele, and both a small deletion (< 50 bp) and insertion (< 30 bp) in the *Wx-B1b* allele of K107 when compared with the wild-type alleles of cv. Chinese Spring. Insertions or deletions were not detected in the *Wx-D1b* allele of Bai-Huo. Mutations detected in the null alleles of stable waxy mutants of maize and rice have been characterized (Wessler and

Varagona 1985; Okagaki and Wessler 1988). In maize, large insertions (150 bp ~ 6.1 kbp) or deletions (> 300 bp) are associated with mutations among the *wx* alleles, while single base changes or very small insertions or deletions were responsible for the mutations in rice. The mechanism of origin of the spontaneous lesions detected in wheat *wx* alleles appears to differ among the three *waxy* loci, and to be more complicated than in maize and rice.

Starch is a major component of the harvested parts of plants and is used in food and non-food industries. Demand for many kinds of natural starch that possess novel physical and chemical properties is increasing, due to the desire to develop new starch products and to reduce the need for post-harvest chemical modification processes (Smith and Martin 1993). A number of naturally occurring mutants with altered proportions of amylose and amylopectin have been well studied (Shannon and Garwood 1984). Of these, waxy maize is widely used in food industries. Waxy maize starch is notable for paste clarity, high water binding capacity and resistance to gel formation and retrogradation (Watson 1988). Starch of amylose-free potatoes yields a similar type of clear paste, which does not retrograde, and seems to be useful in ready-prepared foods (Visser and Jacobsen 1993). Starch of waxy wheats also lacks amylose and may have several applications, including reduction of staling in flour products, especially bread.

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